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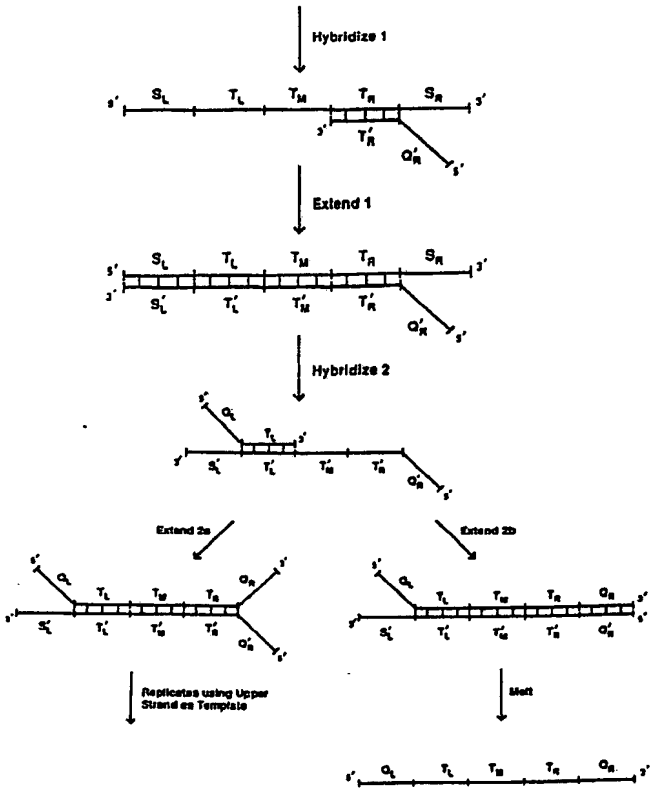
(54) Title: TARGET NUCLEIC ACID AMPLIFICATION/DETECTION SYSTEMS

(57) Abstract

This invention relates to the use of functional reporter molecules in the detection and measurement of RNA sequences in a sample, as a determination, for example, of pathogenic disease existence or potential. The invention is predicated on the utilization of nucleotide sequences, one having a probe sequence linked to a sequence capable of initiating replication by an RNA-dependent RNA polymerase. The other is capable of hybridizing to a strand separated from the extension product of the first nucleotide sequence after hybridization to a specific target sequence. The extension product of the second hybridized nucleotide sequence serves as a template source for autocatalytic replication by the RNA-dependent RNA polymerase. The replication products are detected as a means for detection of nucleic acid sequences.



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TARGET NUCLEIC ACID AMPLIFICATION/DETECTION SYSTEMS

Reference is made to PCT International Application Publication No. WO 87/06270 and PCT International Application No. US89/01966, published November, 1989, the entire disclosures of which are incorporated herein by reference.

Field of the Invention

The present invention relates generally to advances in molecular biology and recombinant DNA technology.

More particularly, the invention is directed to the methods and means, including assays and test kits containing requisite reagents and means, for detecting in an *in vitro* or *ex vivo* setting the presence of target RNA species, and by deduction the corresponding polypeptide that the RNA species encodes, in a biological sample.

The present invention features combining in a novel manner, for use in nucleic acid probe hybridization systems for detection of target RNAs in biological samples, the advantages of target amplification with those of enzymatic production of detectable reporter molecules by using RNA-dependent RNA polymerases to make a copy of an RNA target that incorporates target sequence and is autocatalytically replicatable by the polymerase.

Among the applications in which the present invention finds use are in analyses of RNA sequences characteristic of a disease or pathogenic condition by *in vitro* or *ex vivo* nucleic acid probe hybridization assays of body fluids and tissues containing requisite target RNA.

Background of the Invention

It is a goal in the nucleic acid probe art to detect various nucleic acid sequences in a biological sample, in which said sequences, as so-called target nucleic acid, are present, usually in small amounts relative to the wide variety of other nucleic acid

species, including RNA, DNA or both, present in such a sample. Thus, it is desirable to be able to detect the RNA encoding polypeptides that may be associated with diseases or pathological conditions, such as, for example, RNA of the genome of the human immunodeficiency virus. In addition to the detection of RNAs associated with such viruses, it is desirable to detect other RNAs characteristic of, for example, a disease or pathological condition, such as those transcribed from a defective gene, as in the case of hemophilia or sickle-cell anemia. Characteristically, the RNA associated with such a disease or condition is present, if at all, in very small amounts relative to total nucleic acid in a given biological sample, such as a sample of blood or other body fluid or a tissue sample of an individual to be tested.

The detection of such a RNA species requires such specificity and sensitivity that, if the RNA is present, it is detectable and measurable from among the wide variety of other nucleic acid species with which it is associated in a sample. Some of these non-target nucleic acid species may bear close homology, at least in isolated segments, to the target RNA. Further, as noted above, these target RNA species are often found only in very minute amounts in a biological sample being tested. Yet, for proper diagnosis of the underlying disease state, it is essential that even such small amounts of a target RNA be detectable if present.

Several approaches have been advanced for accomplishing reliable detection, in a biological sample, of a target nucleic acid present, if at all, in only a small amount and as only a minute fraction of total nucleic acid. In one approach, the amount of nucleic acid in the sample is not altered. Instead, a reporter system is employed whereby a large number of readily detectable molecules is produced for each molecule of target nucleic acid in the sample and the presence or

quantity of the detectable molecules is measured. Such a reporter system is a signal-generating system associated with the target nucleic acid and produces a detectable signal representative of the number of molecules of target nucleic acid in a sample.

In another, fundamentally different approach, a target nucleic acid segment, that is part of the target nucleic acid but not other nucleic acids in biological samples to be assayed, or the complement of such a target segment (i.e., the segment with the same size as, but sequence complementary to, that of the target segment), or both the target segment and its complement are selectively increased in copy number. That is, in a sample, the copy number of the target nucleic acid segment or its complement, or the copy numbers of both the target segment and its complement, are increased to a greater extent than the copy number of any other nucleic acid segment. This selective increase in copy number of a nucleic acid segment is referred to in the art as "amplification" of the segment. Once a target segment (also referred to herein, and in the art, as a "target sequence") or the complement of a terget segment is amplified to a sufficient extent, it can be detected reliably by any of many techniques that have been developed in the nucleic acid probe art for the detection of nucleic acid segments, including techniques which involve the first approach, described above, that entails production of many readily detectable molecules for each molecule of target nucleic acid.

One method that has been developed for the amplification of a target segment is the so-called "polymerase chain reaction" ("PCR") method. This technique was reported by Saiki et al., Science 230, 1350 (1985) and Mullis et al., European Patent Application Publication Nos. 200362 and 201184 (See also U.S. Patent Nos. 4683195 and 4683202), and particularly entails repeated cycles of (1) hybridizing to the 3'-end of a

target nucleic acid sequence a first primer and to the 3'-end of the sequence complementary to the target sequence a second primer, (2) extending the primers with a polymerase, and (3) rendering single stranded the duplexes resulting from the chain extension reactions. This PCR procedure results in amplification of the target sequence and its complement exponentially with the number of cycles (i.e., as in a chain reaction).

Certain RNAs are known to be susceptible to autocatalytic replication by certain polymerases, such as bacterial phage RNA-dependent RNA polymerases such as Qβ replicase. These RNAs are said herein to have "template sequences" which means they have sequences that make them templates for replication by the polymerase. In the process, the RNA made from a template RNA (i.e., an RNA with a "template sequence") in the reaction catalyzed by the replicase is also a replicatable RNA (i.e., also has a "template sequence"). Thus, in autocatalytic replication, the amount of replicatable RNA can increase exponentially. See Miele et al., J. Molecular Biology 171, 281 (1983).

Until recently it has not been appreciated that autocatalytic replication could be employed to provide convenient, broadly applicable, highly sensitive reporter systems for analyses of biological samples for the presence of particular nucleic acid sequences. A system in which probe for a target sequence is linked to an RNA capable of being replicated by Qβ replicase is described in PCT International Application Publication No. WO 87/06270 and by Chu et al., Nucleic Acids Research 14, 5591 (1986). Thus, the invention described in the PCT International Application Publication combines the art of replication of RNA with the art of oligonucleotide hybridization probes by providing for detection of target nucleic acid by replicative amplification of replicatable RNA associated (through a probe) with a target segment.

It is an object of the present invention as a

selective embodiment to take further advantage of the basic RNA-replicative process for amplification, for ease in the detection of target RNA sequences, thus achieving exponential copying without the requirement necessarily of temperature cycling. It is a further object of the present invention to take advantage of RNA detection using a process that, in assuring the retention of the target sequence in the amplified product, avoids or at least substantially reduces the presence of false positives because the target is only detected after amplification if it was present in the sample probed. It is a further object of the present invention to combine the advantages of the replicative and extension product procedures as a means for detecting and measuring corresponding target RNA.

It is a basic object of the present invention to employ a first nucleotide sequence bearing a probe sequence and a sequence that is one operatively recognizable by a RNA-dependent RNA polymerase (replicase) for RNA-template-directed, primer initiated RNA strand synthesis (as well as initiation of autocatalytic replication) in conjunction with a second associated nucleotide sequence bearing a second probe sequence and a sequence that is also one operatively recognizable by said RNA-dependent RNA polymerase for RNA-template-directed, primer initiated RNA strand synthesis (as well as initiation of autocatalytic replication), such that an extension product of said first sequence serves as a template for preparation of an extension product of said second sequence, the latter extension product, either as such or in strand disassociated form being replicatable upon appropriate influence of the RNA-dependent RNA polymerase. Thus, the present invention relates to an amplification/detection system that reports only when RNA target sequence is present in the tested sample and whose amplification component operates exponentially without necessity of

temperature cycling. It is thus a preferred object of the present invention to produce, by the initial event of hybridization to an intended, present target RNA sequence, a given extension product that corresponds by presence to target nucleic acid sequence which in turn leads to a subsequent product that is susceptible to amplification by replication to a plurality of RNA segments that in turn can be detected and measured such as via hybridization with an authentic target sequence and/or optionally by association with a signal grouping (i.e., a reporter group that is itself detectable or is capable in a suitable chemical reaction of leading to detectable molecules) that is accountable for their detection and measurement.

It is thus an overall object of the present invention to meet the goals enumerated by the art and to provide selective means to meet disadvantages and problems encountered by prior researchers' endeavors. The present invention utilizes reporter molecules that are present after amplification by replication, only when the necessary target nucleic acid sequence is present in the sample tested. It employs nucleic acid sequences that need only contain a sequence that is susceptible to hybridization potential, chain extension catalyzed by a replicase and ultimately to amplification by autocatalytic replication, and nothing more. Thus, the present invention provides means for detecting target nucleic acid sequences that are responsive only to presence of target RNA sequence itself. It further provides a straightforward technique that can be utilized reproducibly in an acceptably short period of time, employing the convenience of known reagents and having the precision necessary to reach consistent scientific results; one that can be employed in a reproducible assay setting and that is adaptable for use in kits for laboratory/clinical analyses. It is, hence, an object of the present invention to increase the detectability of

certain RNA sequences (target segments) by amplification of sequences associated with the presence of the target sequences in an in vitro or ex vivo system, utilizing in its preferred embodiments the advantages provided by the natural chain extension and replicative processes per se of replicases, and having the unique feature of being measurable only when target nucleic acid sequence is present.

Summary of the Invention

The present invention is predicated on the use in a novel manner of a first nucleotide sequence comprising (i) a probe sequence suitable for hybridization with a segment of a target RNA sequence and (ii) extending in the 5'-direction from the 5'-end of the probe sequence, a first sequence that is a sequence capable of initiating an autocatalytic replication process with a replicase and, thereby also, capable of initiating an RNA-template-directed, RNA primer initiated chain exyension reaction. This first sequence operates herein in conjunction with a second nucleotide sequence, said second nucleotide sequence comprising (i) a second probe sequence suitable for hybridization to the strand-separated extension product of the first nucleotide sequence, at "the opposite end of" the extension product from the first nucelotide sequence (by which is meant, more precisely, at a segment of said extension product 3' from the 3'-end of the first nucleotide sequence, which is at the 5'-end of the extension product), and (ii) and, extending in the 5'-direction from the 5'-end of the second probe sequence, a second sequence that is also a sequence capable of initiating an autocatalytic replication process with a replicase and, thereby also, capable of initiating an RNA-template-directed, RNA primer initiated chain exyension reaction. After hybridization of the first sequence to target sequence, if any, present in a sample being assayed for the target sequence, the first

sequence is extended in a chain extension reaction catalyzed by the replicase. Then the extension product is separated from the target nucleic acid, which comprises the target sequence, and the second sequence is hybridized to the strand-separated extension product and extended in a second chain extension reaction by the replicase. The product of the second extension, as is or in strand-separated form if necessary, is then, in a process hereof of amplification, autocatalytically replicated in the presence of the replicase. The amplified replication products are then detected and measured using any of numerous techniques known per se by the art skilled (see, e.g., PCT International Application Publication No. WO 87/06270).

See Figure 1 hereof for a representative illustration of carrying out the target-sequence-recognition/amplification process aspect of the invention.

In an embodiment, the present invention is directed to novel, cofunctioning nucleotide sequences, their preparation and use, namely the first and second nucleotide sequences just described and described further below.

In another embodiment, the present invention is directed to an extension product of said first nucleotide sequence, made after hybridization of said first nucleotide sequence with said target or an extension product of said second nucleotide sequence and using said target or an extension product of said second nucleotide sequence as template, as well as an extension product of said second nucleotide sequence, made after hybridization of said second nucleotide sequence with the one strand of a strand-separated, first extension product, to which said second nucleotide sequence is capable of hybridizing through the second probe sequence.

The herein mentioned extension products are made in chain extension reactions that are catalyzed by an appropriate RNA-dependent RNA polymerase and carried out

in the presence of the NTPs. In a preferred embodiment, said extension reaction(s) as well as the autocatalytic replication process is (are) conducted with the same replicase, namely, Q-beta replicase, where a sequence recognized by this enzyme is part of the extension product of the second nucleotide sequence hybridized to an extension product of the first nucleotide sequence (hybridized to, e.g., target sequence).

The products of the autocatalytic replication, which will contain replicase-recognizable sequence, are detected and measured in a manner known per se such as, for example, via a chromophore moiety or a radioactive moiety incorporated in the course of the autocatalytic replication or by hybridization with an oligonucleotide probe which is detectably labeled and comprises at least a subsequence of the target sequence or the complement of such a subsequence.

In all respects, the present invention is directed to the novel application of the natural principles of hybridization of complementary RNA sequences, chain extension of RNA primers on RNA templates catalyzed by replicases (see, e.g., Vournakis et al., Biochem. Biophys. Res. Commun. 70. 774 (1976)), and autocatalytic replication of many RNAs, including recombinant RNAs, by replicases (see, e.g., Miele et al., supra; United States Patent No. 4,786,600) in order amplify and render detectable and measureable target RNA sequences of target RNAs that may be present in a biological sample containing a mixture of nucleic acids.

The present invention is further directed to methods and means for assay systems based upon such principles and to kits incorporating components necessary for such assay methodology for detecting or measuring target RNA sequences, including in laboratory and clinical settings.

The present invention thus provides a method for the detection of at least one specific RNA target, in a

sample containing nucleic acid, said RNA target comprising a target sequence and said method comprising detecting replicatable extension product (or complement thereof), said product being the product of extension from a second ribonucleotide (i.e., RNA) sequence hybridized with a first RNA extension product strand-separated from the target RNA, which provides the template for synthesis of the first extension product from a first ribonucleotide sequence hybridized with the 3'-end of the target sequence. In the method, said first ribonucleotide sequence comprises a first probe sequence complementary to a subsequence at the 3'-end of the target sequence and, 5' from and contiguous with (i.e., linked through a single phosphate to) the 5'-terminal ribonucleotide of the probe sequence, a first replication-initiation sequence that (a) is one capable of being recognized by a replicase for initiation of an RNA synthesis (i.e., chain extension) process from the 3'-end of the probe sequence utilizing target RNA sequence as a template and (b) is a part, including the 5'-end, of an RNA that is autocatalytically replicatable by the replicase. Further, said second ribonucleotide sequence comprises (1) a second probe sequence that has the same sequence as a subsequence at the 5'-end of the target sequence (said 5'-subsequence not overlapping the 3'-subsequence of which the first probe sequence is the complement) and, therefore, a sequence that is complementary to that of a subsequence of the extension product from the first nucleotide sequence hybridized to target RNA, the 5'-end of said subsequence being 3' from the 3'-end of the first nucleotide sequence in said extension product; and (2) 5'-from and contiguous with said second probe sequence, a second replication-initiation sequence that (a) is one capable of being recognized by the replicase for initiation of an RNA synthesis (i.e., chain extension) process from the 3'-end of the second probe sequence utilizing complement of

target RNA sequence as a template and (b) is a part, including the 5'-end, of an RNA that is autocatalytically replicatable by the replicase, provided that the RNA which consists of the second replication-initiation sequence joined at its 3'-end through a single phosphate to the 5'-end of the complement of the first replication-initiation sequence is autocatalytically replicable by the replicase and provided further that an RNA which is made from said autocatalytically replicable RNA by inserting therein, between the 3'-end of said second replication-initiation sequence and the 5'-end of the complement of said first replication initiation sequence, an RNA segment is also autocatalytically replicable by the replicase. The replicatable extension product of said second nucleotide sequence functions, by autocatalytic replication catalyzed by the replicase, to amplify the target sequence. The replicatable extension product of the second nucleotide sequence as well as the complement of said extension product, by virtue of being autocatalytically replicated, also serve as reporter molecules for the target sequence and associated target RNA.

The present invention advantageously combines the use of replication (i.e., autocatalytic replication), thus providing selective, exponential growth of the replicated nucleic acids without temperature cycling required in processes such as PCR, with inclusion of the target RNA sequence in the replicatable product, thereby providing amplification of the target sequence, rather than simply a number of reporter molecules for each molecule of target. The present invention also advantageously utilizes the capability of RNA-dependent RNA-polymerases, such as Q-beta replicase, to both synthesize RNAs from a primer using an RNA as a template and autocatalytically replicate RNAs, thus providing for amplification of target and production of reporter molecules with a single enzyme.

The present invention further embodies means for detecting and measuring the amount of the products of the autocatalytic replication and, thereby, also measuring the amount of target RNA present in a sample being analyzed.

In an aspect, the present invention is directed to a method useful for the detection of at least one specific RNA target sequence in a sample containing nucleic acid, comprising:

hybridizing with said target RNA sequence under suitable conditions a nucleotide sequence comprising a probe sequence corresponding in sequence to a segment of said target sequence and a functional length of sequence that is the complement of one susceptible to replication upon association with an appropriate RNA-dependent RNA polymerase,

chain extending said hybridized nucleotide sequence,

strand separating the extension product,

hybridizing with the strand separated in the previous step and containing the sequence that is the complement of one susceptible to replication a second nucleotide sequence comprising a sequence capable of hybridizing with said separated strand at the end opposite of the sequence that is the complement of said target sequence and a functional length of sequence that is the complement of one susceptible to replication upon association with an appropriate RNA-dependent RNA polymerase,

chain extending said hybridized second nucleotide sequence,

permitting operatively the second extension product of the previous step, optionally after strand separation, to undergo replication by contact with an appropriate RNA-dependent RNA polymerase, and

detecting the replication products.

The present invention, in application, embodies the detection of said amplification products such as via radio- or chromophore-labeling or hybridization techniques known per se.

The present invention contemplates the detection of RNA target sequence in a sample wherein said target sequence is one associated with characteristics of a genetic or pathogenic disease or condition, and particularly those wherein the target RNA sequence is a segment of RNA of a human virus or a transcript of a defective gene or a defective transcript of a normal gene.

There are a number of human diseases that are either the direct result of a genetic defect or are correlated with the presence of a particular genetic allele. By way of example, the technique described in this application could be used to determine whether or not a given target is present in a very small sample of nucleic acid. This would be useful in the diagnosis of genetic disorders via the detection of corresponding mRNA species or in the testing for presence of viral infection, e.g., HIV-1.

The present invention contemplates the use of appropriate RNA-dependent RNA polymerase (replicase) enzymes that are capable both of chain extension and replication. A preferred embodiment employs Q-beta replicase enzyme to achieve both functions in a convenient, so-called single-pot reaction.

The present invention is also directed to assay systems and kits embodying same, useful for the detection of at least one specific RNA target sequence in a sample containing nucleic acid, after amplification of the target RNA sequence in accordance with the present invention. Kits would comprise a replicase, a first nucleotide sequence for carrying out the amplification process, as described above, a second nucleotide sequence for carrying out the amplification process, as described

above, and compositions required to detect or provide detectability to the amplified product.

Detailed Description of the Invention

1. Brief description of the drawing

Figure 1 depicts schematically an aspect of this invention, namely the steps hereof in target amplification using a single- or double-stranded RNA template for replication. Extension and amplification are carried out, e.g., with Q-beta RNA polymerase (replicase). T(target) is redesignated in segmented fashion as $T_L T_M T_R$. The sequences $Q_L T_L$ and $T_R' Q_R'$ may be obtained by molecular cloning and subsequent in vitro transcription. The required template for autocatalytic replication amplification is obtained by hybridization, extension, rehybridization and reextension as shown. Where the Q sequences represent Q-beta sequences for initiation of chain extension and replication, Q-beta replicase both chain extends and replicates, in turn.

2. General methods and definitions

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques of the present invention, such as:

RNA probe or primer preparation, including transcription of encoding DNA in an expression vector and the tailoring thereof so as to be suitable as such or when linked to other RNA for use as a probe herein;

preparation of nucleotides with different functional sequences for use in hybridization;

hybridization methodology including variations in stringency conditions for producing more or less hybridization certainty depending on the degree of homology of the primer to a target RNA sequence;

identification, isolation or preparation of RNA polymerases capable of chain extension reactions and of recognizing said replicatable sequences referred to above;

conditions conducive to the initiation and maintenance of extension reactions including use of RNA-dependent RNA polymerase and NTPs;

the mechanism and methodology for autocatalytic (sometimes referred to herein as "induced") replication; and so forth.

See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York 1982), and Colowick et al., Methods in Enzymology Volume 152, Academic Press, Inc. (1987), and the various references cited therein.

All of the aforecited publications are by this reference hereby expressly incorporated herein.

By the term "probe" in the present context is meant a RNA sequence that has sufficient homology with the target sequence such that under suitable hybridization conditions it is capable of hybridizing, that is binding to, the target sequence. A typical probe is at least about 10 nucleotides in length, and most preferably is of approximately 25 or more nucleotide bases in length, and in its most preferred embodiments, it shares identity or very high homology with the target sequence. See, for example, EPA 128042 (publd. 12 Dec 84).

The techniques of forming a detection signal such as via radioactive labeling or chromogenic means using a chromogenic susceptible enzyme are also well known and documented in the art.

A sample on which the assay method of the invention is carried out can be a raw specimen of biological material, such as serum or other body fluid, tissue culture medium or food material. More typically, the method is carried out on a sample which is a

processed specimen, derived from a raw specimen by various treatments to remove materials that would interfere with detection of target, such as by causing non-specific binding of affinity molecules. Methods of processing raw samples to obtain a sample more suitable for the assay methods of the invention are well known in the art.

Reference herein to bacteriophage Qβ is not limited to any particular variant or mutant or population thereof. Such reference, unless otherwise specifically limited, is to any variant, mutant or population which, upon infection therewith of E. coli susceptible to bacteriophage Qβ infection, is capable of causing production of an RNA-dependent RNA-polymerase or any polymerase acting as a replicase and its associated nucleic acid substrate.

For other phages which, upon infection of bacteria susceptible to infection therewith, produce RNA-dependent RNA polymerases, and associated replicatable RNAs capable of being autocatalytically replicated in vitro, which can be employed in the present invention, see, e.g., Miyake et al., Proc. Natl. Acad. Sci. (U.S.A.) 68, 2022 (1971).

RNA resulting from the replication process can be made fluorescent by employing a T4 RNA ligase catalyzed reaction to append nucleotides modified to be fluorescent to the 3'-end of replicative RNA. See Cosstick et al., Nucl. Acids Res. 12, 1791 (1984). The fluorescence of the resulting RNA can be employed to detect the RNA by any of several standard techniques.

Among still other methods that can be used to detect replicated RNA are those wherein a reporter substance, that binds specifically with nucleic acid, is added to the system in which the replication has taken place, or to the medium, such as a positively charged support such as ECTEOLA paper, on which replicated RNA has been isolated, and signal from the reporter substance measured. Such substances include: chromogenic dyes,

such as "stains all" (Dahlberg et al., J. Mol. Biol. 41, 139 (1969); methylene blue (Dingman et al., Biochemistry 7, 659 (1968), and silver stain (Sammons et al., Electrophoresis 2, 135 (1981); Igloi, Anal. Biochem. 134, 184 (1983)); fluorogenic compounds that bind to RNA -- for example, ethidium bromide (Sharp et al., Biochemistry 12, 3055 (1973); Bailey et al., Anal. Biochem. 70, 75 (1976); and fluorogenic compounds that bind specifically to RNAs that are templates for replication by Qβ replicase -- for example, a phycobiliprotein (Oi et al., J. Cell Biol. 93, 981 (1982); Stryer et al., U.S. Patent No. 4,520,110) conjugated to the viral subunit of Qβ replicase.

Provided that the concentration of replicase remains above the concentration of template RNA, and that ribonucleoside-5'-triphosphate concentration does not become limiting, the concentration of template RNA will increase exponentially with time during replicase-catalyzed RNA replication. After template RNA concentration equals or exceeds replicase concentration, as long as ribonucleoside-5'-triphosphate concentration does not become limiting, the concentration of template RNA will increase linearly with time. See, e.g., Kramer et al. (1974), supra.

It has been found that, under the conditions for replicase-catalyzed replication, the MDV-1 RNA there exemplified doubled in concentration every 36 seconds, until template concentration exceeded enzyme concentration.

The concentration of template RNA, in a replicase-catalyzed replication reaction system after a given time for reaction, will be related to the initial concentration of template RNA. If, at all times during the replication reaction, the concentration of replicase exceeds that of template (and ribonucleoside-5'-triphosphate concentration does not become limiting), the log of concentration of template RNA at the conclusion of

the reaction will be directly proportional to the log of the initial concentration of template (at the start of the reaction). After replicase concentration falls below template concentration, as long as ribonucleoside-5'-triphosphate concentration does not become limiting, the concentration of template at the conclusion of reaction is directly proportional to the log of the initial concentration of template. Further, the time required for a reaction to reach the point at which template concentration equals replicase concentration is proportional to the negative log of the initial concentration of template.

By allowing the replication reaction to proceed for longer times, greater sensitivity can be achieved.

In assays according to the invention, assays are carried out simultaneously, under conditions as nearly alike as possible, on both test samples, which are being tested for target, and control samples. As understood in the art, control samples are similar to test samples but are known to contain either no target or a known quantity of target. A control with no target establishes the "background," below which it is not possible to distinguish samples which contain target from those which do not. By comparing the amount or concentration of replicated replicative RNA produced in an assay of a test sample with the amount or concentration produced with control samples assayed simultaneously, the presence of target in test sample at a level above background can be determined. If control samples with a range of known concentrations of target are employed, the concentration of target in a test sample can be estimated.

Again, the use of a "replicase" for (autocatalytic) induction of replication of the RNA products of the present invention are generally known in the art. Suitable examples of such replicases that are useful in the present invention include the so-called Qβ virus replicase that recognizes a certain nucleic acid

sequence sites at the 3'-end of the given RNA transcript. These replicases serve to replicate, that is reproduce, the RNA transcripts and complements so as to multiply copies thereof. When such enzyme is present in the reaction locus during the process of transcription, it can be foreseen that the multiple transcripts that are produced during transcription can themselves undergo replication so as to exponentially increase the amount of RNA transcript product.

The following examples illustrate a model system of this invention:

4. Examples

Exemplified is the use of Q-beta polymerase and an RNA substrate that is replicatable by said polymerase in order to amplify a target RNA sequence that is contained within the RNA substrate. Q-beta replicase is an RNA-dependent RNA polymerase that recognizes characteristic structural elements at the 3' end of an RNA substrate and subsequently produces a complementary copy of the substrate. If the complementary copy also has the requisite structural elements at its 3' end, then it too can be recombinized and copied by Q-beta replicase, resulting in an autocatalytic reaction cycle that exponentially amplifies the substrate sequence

Q-beta replicase is able to bypass its normal initiation specificity and extend complementary synthesis from the 3' end of a suitable oligonucleotide primer (Felix, G. & Hake, H. Biochem. Biophys. Res. Comm. 65, 503-509, 1975). The reaction generates mainly partial-length cRNAs and a considerable amount of non-specific RNAs (Vournakis et al., Biochem. Biophys. Res. Comm. 70, 774-782, 1976), and thus is not suitable for replication. However, it could be used to extend an RNA primer through a short target region of about 20-100 nucleotides.

Preparation of Primers

Two RNA primers are prepared by in vitro transcription of a suitably constructed recombinant DNA. The first primer contains the first 157 nucleotides (at the 5'-end) of the minus-strand of Q-beta MDV-1 RNA followed by 10 - 50 nucleotides that are complementary to the target RNA over a region extending 5' from the 3' end (i.e. "just downstream from") of the site of interest (i.e., the target sequence). The second primer contains the first 61 nucleotides (at the 5' end) of the plus-strand of Q-beta MDV-1 RNA followed by 10-50 nucleotides that are identical to the target RNA over a region extending 3' from the 5'-end of the target sequence.

Hybridization and Primer Extension

A control template DNA, such as pT7-0 (U.S. Biochemical), is used to prepare suitable RNA transcripts that can serve as a target for detection. 1 fg, 10 fg, 100 fg, 1 pg, 10 pg, or 100 pg of the RNA transcript (10^{-5} fmol - 10^{-3} pmol) is diluted to 50 µl volume to give a final solution containing 100 mM Tris·HCl (pH 7.5), 22 mM $MgCl_2$, 2 mM Na_2EDTA, and 1 mM (each) of the four NTPs. To this solution is added a 25 µl volume containing 2 ng (approx. 1 nM) of each of the two RNA primers. The mixture is heated to 70°C for 1 min and then quick-cooled on ice. A 25 µl volume containing 2 µg of Q-beta replicase is added, and the mixture is incubated at 37°C for 10 min. The mixture is again heated to 70°C for 1 min and quick-cooled on ice. A second 25 µl volume containing 2 µg of Q-beta replicase is added, and the mixture is again incubated at 37°C for 10 min.

Amplification of Target RNA

The second primer-extension product is optionally released from the template by heating to 70°C for 1 min and quick-cooling on ice. If this step is included, then a third 25 µl volume containing 2 µg of Q-beta replicase in a solution containing 50 mM Tris·HCl (pH 7.5), 11 mM $MgCl_2$, 1 mM Na_2EDTA, and 0.5 mM (each) of the four NTPs must be added. In either case, amplification of the

target RNA then proceeds autocatalytically by incubating at 37°C for 20 min. The resulting mixture can then be assayed for the production of MDV-1 RNA which contains an insert that corresponds to the desired target sequence.

Detection of Replicated RNA

The amount of RNA is determined by its intrinsic UV absorbance (e.g. as by the contact photoprinting method of Kutateladze et al., Anal. Biochem. 100, 129 (1979)). Alternatively, the RNA is visualized on ETEOLA paper. Aliquots (of equal volume) of replication reaction are transferred with 13, 48 or 96-fingered aliquotter to sheets of diethylaminoethyl cellulose paper. The sheets are then washed at room temperature in a solution of 200 mM NaCl, 300 mM ammonium acetate pH 6 to remove ribonucleoside triphosphates not incorporated into RNA. The sheets are then stained with 0.3 μg/ml of ethidium bromide. (Sharp et al., Biochemistry 12, 3055 (1973); Bailey et al., Anal. Biochem 70, 75 (1976).

Finally the fluorescence from individual blots is measured by any of several known techniques. Fluorescence intensity from a stained blot above that from control blots indicates the presence of target. Other staining materials can be employed in place of ethidium bromide. These include methylene blue (Dingman and Peacock, Biochemistry 7, 659 (1968)), silver stain (Sammons, et al., Electrophoresis 2, 135 (1981)) or phycobiliprotein Qβ replicase conjugate (Oi et al., J. Cell Biol. 93, 981 (1982)).

The foregoing description details more specific methods that can be employed to practice the present invention and represents the best mode contemplated. However detailed the foregoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

Claims:

1. Cofunctioning nucleotide sequences;

a first nucleotide sequence comprising:

(1) a first probe sequence capable of hybridizing to a target RNA sequence in a sample containing same and

(2) a sequence that is the complement of one recognizable by a RNA-dependent RNA polymerase;

a second nucleotide sequence comprising:

(1) a second probe sequence capable of hybridizing to the end opposite the sequence of said first nucleotide sequence of the strand separated extension product of said first nucleotide sequence and

(2) a sequence that is the complement of one recognizable by a RNA-dependent RNA polymerase.

2. Nucleotide sequences according to Claim 1 wherein said RNA-dependent RNA polymerase is Q-beta replicase.

3. Nucleotide sequences according to Claim 1 or 2 wherein said target sequence is a RNA segment corresponding to a human immunodeficiency virus.

4. Nucleotide sequences according to Claim 1 or 2 wherein said target sequence is a transcript of a defective gene or a defective transcript of a normal gene.

5. An extension product of the first nucleotide sequence according to Claim 1 after its hybridization with the target sequence.

6. An extension product of the second nucleotide sequence according to Claim 1 after its hybridization with the separated strand of the first extension product bearing the sequence corresponding to said first nucleotide sequence.

7. A method useful for the detection of at least one specific RNA target sequence in a sample containing nucleic acid, comprising detecting replicatable extension product, said product being the product of extension from a second nucleotide sequence hybridized with a strand separated from a first extension product that contains a sequence of a first nucleotide sequence hybridizable with a target RNA sequence, said replicatable extension product functioning as a reporter molecule for said target, said first nucleotide sequence comprising a probe sequence complementary to said target sequence and a sequence that is the complement of one capable of initiating a replication process, said second nucleotide sequence comprising a probe sequence complementary to the opposite end of the strand separated from the first extension product bearing the sequence of said first nucleotide sequence such that the extension product of the second nucleotide sequence serves as a template source for replication.

8. A method according to Claim 1 including the additional step in detecting replicatable product by permitting said product to replicate to a plurality.

9. A method according to Claim 8 wherein said replication is effected by contacting replicatable product with replicase enzyme.

10. A method according to Claim 9 wherein said replicase enzyme is Q-beta replicase.

11. A method useful for the detection of at least one specific RNA target sequence in a sample containing nucleic acid, comprising:

hybridizing with said target RNA sequence under suitable conditions a nucleotide sequence comprising a probe sequence corresponding in sequence to a segment of said target sequence and a functional length of sequence that is the complement of one susceptible to replication upon association with an appropriate RNA-dependent RNA polymerase,

chain extending said hybridized nucleotide sequence,

strand separating the extension product,

hybridizing with the strand separated in the previous step and containing the sequence that is the complement of one susceptible to replication a second nucleotide sequence comprising a sequence capable of hybridizing with said separated strand at the end opposite of the sequence that is the complement of said target sequence and a functional length of sequence that is the complement of one susceptible to replication upon association with an appropriate RNA-dependent RNA polymerase,

chain extending said hybridized second nucleotide sequence,

permitting operatively the second extension product of the previous step, optionally after strand separation, to undergo replication by contact with an appropriate RNA-dependent RNA polymerase, and

detecting the replication products.

12. The method according to Claim 11 wherein said replicase enzyme is Q-beta replicase.

13. The method according to Claim 11 or 12 wherein the detected products are measured in a standardized manner so as to measure the amount of target sequence contained in a sample of nucleic acid.

14. The method according to Claim 11, 12 or 13 wherein said target sequence is disposed within a nucleic acid sequence associated with the characteristics of a genetic or pathogenic disease or condition.

15. The method according to Claim 14 wherein said nucleic acid sequence is a RNA segment corresponding to a human immunodeficiency virus.

16. The method according to Claim 14 wherein said nucleic acid sequence is a transcript of a defective gene or a defective transcript of a normal gene.

17. The method according to Claim 11 or 12 wherein said detected products are labeled prior to detection.

18. The method according to Claim 17 wherein said products are radio-labeled.

19. The method according to Claim 17 wherein said products are chromophore labeled.

20. The method according to any one of Claims 11 to 16 wherein said detecting is conducted by hybridization of the replicated products with an authentic, optionally labeled, sequence of target sequence.

21. A kit useful for the detection of at least one specific RNA target sequence in a sample containing nucleic acid, comprising detecting replicatable extension product, said product being the product of extension from a second nucleotide sequence hybridized with a strand separated from a first extension product that contains a sequence of a first nucleotide sequence hybridizable with a target RNA sequence, said replicatable extension product functioning as a reporter molecule for said target, said first nucleotide sequence comprising a probe sequence complementary to said target sequence and a sequence that is the complement of one capable of initiating a replication process, said second nucleotide sequence comprising a probe sequence complementary to the opposite end of the strand separated from the first extension product bearing the sequence of said first nucleotide sequence such that the extension product of the second nucleotide sequence serves as a template source for replication, and means for hybridizing said nucleotide sequences and for chain extending said hybridized nucleotide sequences and for amplifying by replication said extension product and for detecting and optionally measuring the replication products therefrom, and by deduction said target sequence.

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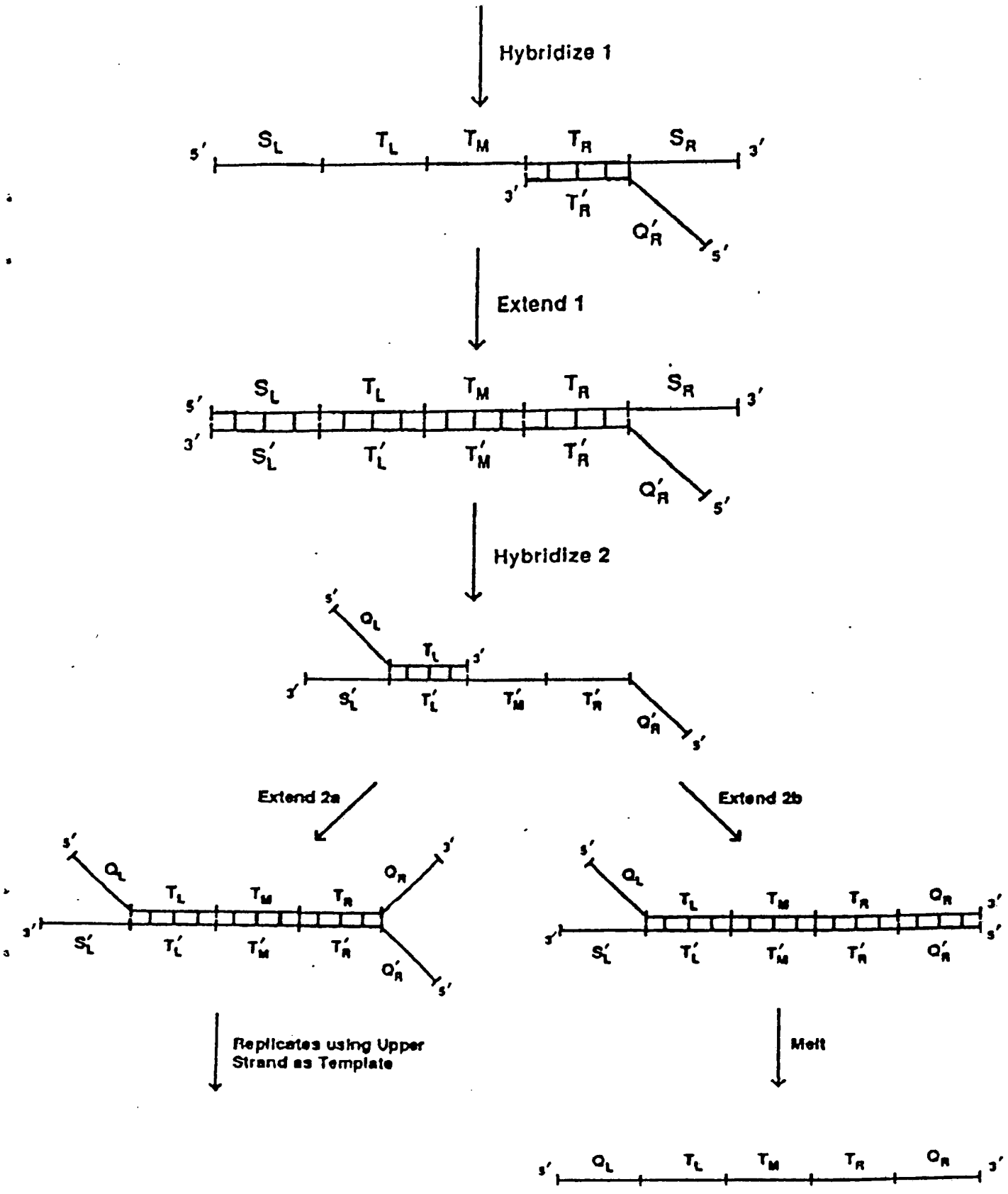
FIG. 1


Hybridize 1
Extend 1
Hybridize 2
Extend 2a
Extend 2b
Replicates using Upper Strand as Template
Melt

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05533

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68
U.S. Cl.: 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/91,288,810 436/501,808,811 536/27 935/3,17,77,78,86,88

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Journal of Molecular Biology, Volume 171, published in 1983, E. A. Miele et al., "Autocatalytic Replication of a Recombinant RNA," pages 281-295, see especially the Abstract on page 281, Figure 1 on page 286, and the bridging paragraph on pages 293-294.	1,2,4-14, 16-21
X	Nucleic Acids Research, Volume 14, Number 14, published in 1986, B. C. F. Chu, et al., "Synthesis of an amplifiable RNA for bioassays," pages 5591-5603, see especially page 5602, lines 7-22.	1,2,4-14, 16-21
Y	Biochemical and Biophysical Research Communications, Volume 65, Number 2, published in 1975, G. Feix et al., "Primer Directed Initiation of RNA Synthesis Catalysed by QB Replicase," pages 503-509, see especially page 503, the Abstract plus the next 12 lines.	1-21

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 MARCH 1990 (20.03.90)	30 MAR 1990
International Searching Authority	**Signature of Authorized Officer [14]**
ISA/US	ARDIN MARSCHEL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Biochemical and Biophysical Research Communications, Volume 70, Number 3, published in 1976, J. Vournakis et al., "Synthesis of RNA Complementary to Rabbit Globin mRNA by QB Replicase," pages 774-782, see especially the Conclusions on page 781.	1-21
X	US, A, 4,786,600, KRAMER ET AL., 22 NOVEMBER 1988 (22.11.88), see the Abstract and claim 9.	1,2,4-14, 16-21
Y	US, A, 4,683,202, MULLIS, 28 JULY 1987 (28.07.87), see the Abstract and claims 1-3, 6, 10, and 11.	1-21